

Remarks

Further and favorable reconsideration of the subject application in light of the following remarks and pursuant to 37 C.F.R. § 116, are respectfully requested. Claims 1, 10-12 and 42-45 are currently under consideration. Claims 43-45 are allowed. Claims 46-48 are new.

Objection to Claims 11, 12, and 42

The Examiner has objected to claims 11, 12, and 42 as being dependent upon a rejected base claim, but as allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims. Applicants have submitted such rewritten claims as new claims 46-48 and respectfully submit that they are in condition for allowance.

Rejection under 35 U.S.C. § 112, Para. 2

Applicants acknowledge that the Examiner has withdrawn the rejection of Claims 10-12 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention.

Rejection under 35 U.S.C. § 112, Para. 1

Claims 1 and 10 remain rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. The Examiner maintains that "Applicant has not transformed plants with the Arabidopsis putative tocopherol cyclase gene, or evaluated the ability of this gene to either encode tocopherol cyclase or to restore tocopherol cyclase to knockout mutants....In the absence of a definite function, Applicant has not taught how to make and use the invention as broadly claimed." (*Office Action at 3.*)

Applicants respectfully disagree that Applicants have not enabled one of ordinary skill in the art to make and/or use the invention. The sequence listing sets forth SEQ ID NO: 109, the slr1737 *Arabidopsis* homologue and SEQ: ID NO: 110, the protein. Applicants submit herewith a protein alignment between SEQ ID NO: 110 and the sequence used in the work reported in Porfirova, PNAS Sept. 17, 2002, vol. 99, no. 19, 12495-12500(GI:24212569). This protein alignment shows that the sequences are identical. (See attachment 1).

Porfirova states that:

The gene at4g32770 on BAC F4D11 (Gen Bank accession no. AL022537) represented a candidate gene for VTE1, because it encodes a protein similar to the ORF slr1737 of *Synechocystis* (52.5% and 41.1% identity on DNA and amino acid level, respectively). In *Synechocystis*, slr1737 is localized in the same operon as slr1736, which encodes the homogentisate phytyltransferase, another enzyme of tocopherol synthesis (citation omitted). Because in prokaryotes, genes encoding enzymes of the same biosynthetic pathway are oftentimes combined in operons, the

ORF slr1737 was a likely candidate for another gene of tocopherol synthesis...For this reason, the gene At4g32770 from *Arabidopsis* was amplified by PCR from genomic DNA of *vte1* and completely sequenced. (Id. at 12497.)

...
To provide further evidence that the VTE1 protein of *Arabidopsis* codes for a functional enzyme with tocopherol cyclase activity, the corresponding cDNA was heterologously expressed in *E. coli* and used for enzyme assays....As depicted in Fig. 5, the VTE1 protein showed high tocopherol cyclase activity with the substrates DMPQ and DMGQ, resulting in the synthesis of γ -tocopherol and γ -tocotrienol, respectively. These findings provide clear evidence that *VTE1* encodes tocopherol cyclase and that this enzyme is involved in the synthesis of tocopherols. (Id. at 12497-12498)

Applicants respectfully submit that the function of the gene which applicants identified has been confirmed, and thus, that the rejection under 35 U.S.C. § 112, first paragraph, should be withdrawn.

Rejection under 35 U.S.C. § 112, Para. 1

Claims 1 and 10 remain rejected under 35 U.S.C. Section 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

The Examiner asserts that the "putative *Arabidopsis* tocopherol cyclase sequence of SEQ ID NO: 109, a sequence taken from a BAC clone, does not support the description of a genus of tocopherol cyclase encoding polynucleotides because the specification and the prior art do not show any correlation between the *Arabidopsis* BAC sequence and the *Synechocystis* sequence, and the claimed prokaryotic unifunctional tocopherol cyclase activity and thus, Applicant has described only one tocopherol cyclase". (*Office Action at 2.*) Applicants respectfully disagree with the Examiner's reasoning and conclusion. The application sets forth at Example 6E, p. 48, that the *arabidopsis* homologue was found using a BLASTALL search using a *Synechocystis* sp gene slr1737 as the query. (Further, the application describes how the homologue sequenced differed in the private database from that found in the public database). Further, as described above, the function of the *arabidopsis* homologue has been demonstrated.

For these reasons and the additional reasons set forth in the Response to the Office Action Mailed December 18, 2002, applicants respectfully submit that more than one tocopherol cyclase has been described and further than the subject matter of claims 1 and 10 is described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor(s), at the time the application was filed, had possession of the claimed invention. Thus, the rejection should be withdrawn.

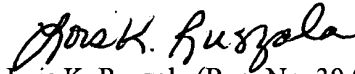
It is not believed that extensions of time or fees for net addition of claims are required beyond those that may otherwise be provided for in the documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any fees required therefor (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account Number 50-1100,

Appl. No 09/688,069
Amdt dated 4 Sep 03
Resp to Final OA of June 3, 2003
Atty Ref: REN1-00-091-US

referencing docket number REN1-00-091. Applicants likewise authorize a charge to Deposit Account Number 50-1100 for any other fees related to the present application that are not otherwise provided for in the accompanying documents.

Should the Examiner have any questions regarding this application, the Examiner is encouraged to contact Applicants' undersigned representative at (847) 457 5055.

Respectfully submitted,



Lois K. Ruzala (Reg. No. 39,074)

Donald J. Silvert (Reg. No. 37,552)

Thomas E. Omholt (Reg. No. 37,052)

Renessen LLC
Legal Dept - Intellectual Property
3000 Lakeside Dr., #300-South
Bannockburn, IL 60015
847-457-5130 (phone contact)
847-457-5174 (fax)

Plurality: 2.00 Threshold: 4 AveWeight 1.00 AveMatch 2.78 AvMismatch
-2.25

Symbol comparison table: blosum62.cmp CompCheck: 1102

GapWeight: 8
GapLengthWeight: 2

PileUp MSF: 488 Type: P November 18, 2002 15:14 Check: 8331 ..

Name: sl1737ArabMo Len: 488 Check: 2777 Weight: 1.00
Name: slr1737ArabPoriferova Len: 488 Check: 2777 Weight: 1.00

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	1		50
sl1737ArabMo	MEIRSLIVSM NPNLSSFELS RPSPLTRSL VPFRSTKLVP RSISRVSASI		
slr1737ArabP	MEIRSLIVSM NPNLSSFELS RPSPLTRSL VPFRSTKLVP RSISRVSASI		
	51		100
sl1737ArabMo	STPNSETDKI SVKPVYVPTS PNRELRTPHS GYHFDGTPRK FFEGWYFRVS		
slr1737ArabP	STPNSETDKI SVKPVYVPTS PNRELRTPHS GYHFDGTPRK FFEGWYFRVS		
	101		150
sl1737ArabMo	IPEKRESFCF MYSVENPAFR QSLSPLEVAL YGPRFTGVGA QILGANDKYL		
slr1737ArabP	IPEKRESFCF MYSVENPAFR QSLSPLEVAL YGPRFTGVGA QILGANDKYL		
	151		200
sl1737ArabMo	CQYEQDSHNF WCDRHLEVLG NTFSAVPGAK APNKEVPPEE FNRRVSEGfq		
slr1737ArabP	CQYEQDSHNF WCDRHLEVLG NTFSAVPGAK APNKEVPPEE FNRRVSEGfq		
	201		250
sl1737ArabMo	ATPFWHQCHI CDDGRTDYAE TVKSARWEYS TRPVYCWGDV GAKQKSTAGW		
slr1737ArabP	ATPFWHQCHI CDDGRTDYAE TVKSARWEYS TRPVYCWGDV GAKQKSTAGW		
	251		300
sl1737ArabMo	PAAFPVFEPH WQICMAGGLS TGWIEWGGER FEFRDAPSYS EKNWGGGFPR		
slr1737ArabP	PAAFPVFEPH WQICMAGGLS TGWIEWGGER FEFRDAPSYS EKNWGGGFPR		
	301		350
sl1737ArabMo	KWFWVQCNVF ECATGEVALT AGGGLRQLPG LTETYENAAL VCVHYDGKMY		
slr1737ArabP	KWFWVQCNVF ECATGEVALT AGGGLRQLPG LTETYENAAL VCVHYDGKMY		
	351		400
sl1737ArabMo	EFVPWNGVVR WEMSPWGYWY ITAENENHVV ELEARTNEAG TPLRAPTTEV		
slr1737ArabP	EFVPWNGVVR WEMSPWGYWY ITAENENHVV ELEARTNEAG TPLRAPTTEV		
	401		450
sl1737ArabMo	GLATACRDSC YGELKLQIWE RLYDGSKGKV ILETKSSMAA VEIGGGPWFG		
slr1737ArabP	GLATACRDSC YGELKLQIWE RLYDGSKGKV ILETKSSMAA VEIGGGPWFG		
	451		488
sl1737ArabMo	TWKGDTSNTP ELLKQALQVP LDLESALGLV PFFKPPGL		
slr1737ArabP	TWKGDTSNTP ELLKQALQVP LDLESALGLV PFFKPPGL		

Isolation of an *Arabidopsis* mutant lacking vitamin E and identification of a cyclase essential for all tocopherol biosynthesis

Svetlana Porfirova*, Eveline Bergmüller*, Susanne Tropf†, Rainer Lemke†, and Peter Dörmann**

*Department of Lothar Willmitzer, Max Planck Institute of Molecular Plant Physiology, Am Mühlenberg 1, 14476 Golm, Germany; and †SunGene GmbH & Co. KGaA, Corrensstrasse 3, 06466 Gatersleben, Germany

Edited by Elisabeth Gantt, University of Maryland, College Park, MD, and approved July 16, 2002 (received for review June 3, 2002)

Tocopherol (vitamin E) is a plant chloroplast lipid presumed to be involved in the response to oxidative stress. A tocopherol-deficient mutant (*vte1*) was isolated from *Arabidopsis thaliana* by using a TLC-based screening approach. Mutant plants lacked all four tocopherol forms and were deficient in tocopherol cyclase activity. Genetic mapping of *vte1* and a genomics-based approach led to the identification of the ORF At4g32770 as a candidate gene for tocopherol cyclase. In *vte1*, At4g32770 contains a splicing site mutation and the corresponding mRNA expression is reduced. Expression of VTE1 in *Escherichia coli* resulted in the production of a protein with high tocopherol cyclase and tocotrienol cyclase activity. The VTE1 sequence shows no similarities to genes with known function, but is similar to that of *SXD1*, a gene that was recently isolated based on the availability of the *sucrose export defective1* maize mutant (*sxd1*). Growth of the *vte1* mutant, chlorophyll content, and photosynthetic quantum yield were similar to wild type under optimal growth conditions. Therefore, absence of tocopherol has no large impact on photosynthesis or plant viability, suggesting that other antioxidants can compensate for the loss of tocopherol. During photo-oxidative stress, chlorophyll content and photosynthetic quantum yield were slightly reduced in *vte1* as compared with wild type indicating a potential role for tocopherol in maintaining an optimal photosynthesis rate under high-light stress.

During their life cycle, higher plants are exposed to a variety of abiotic and biotic stress factors, including high temperature, drought, high light, and senescence. Under these stress conditions, reactive oxygen species derived from molecular oxygen (peroxide, hyperoxide, hydroxyl radicals, singlet oxygen) can accumulate in the leaves resulting in the oxidation of many important cellular components, including proteins, chlorophyll, and lipids, and may finally result in cell death (e.g., refs. 1–4). Different biochemical redox systems participate in the plant's response to oxidative stress—e.g., ascorbate, glutathione, salicylate, and tocopherol (5–8). Furthermore, transcription of stress-related genes is induced, resulting in the expression of enzymes involved in scavenging reactive oxygen species (9, 10). The role of the stress-induced accumulation of specific lipids is only poorly understood (11–13). In contrast to galactolipids and phospholipids, which are abundant in plant membranes, different lipids (triacylglycerol, free fatty acids, and tocopherol) accumulate in the leaves during stress.

Tocopherol represents an amphiphilic lipid that was proposed to be crucial for the oxidative stress response in plant membranes (5). The hydrophilic chromanol ring that is localized close to the lipid/water interface of membranes encompasses a hydroquinone ring system capable of undergoing redox reactions with reactive oxygen species (5, 14). Based on the number and position of methyl groups on the hydroquinone ring, four forms of tocopherol can be distinguished (α -, β -, γ -, δ -tocopherol). In addition to tocopherols, tocotrienols are found in small amounts in plant tissues. Tocotrienols carry an unsaturated geranylgeranyl side chain bound to the chromanol ring instead of the

saturated phytyl chain. Their exact function in plants is unknown. In *in vitro* experiments, tocopherol was shown to be responsible for scavenging reactive oxygen species, thereby preventing the oxidative degradation of fatty acids in membranes (15–17). Because tocopherol is particularly enriched in chloroplast membranes (18, 19), it was proposed to be involved in the protection of chloroplast lipids and of chlorophyll against oxidative damage.

The eight different forms of tocopherol and tocotrienol (commonly referred to as vitamin E) represent an essential component of the human diet, with α -tocopherol showing the highest vitamin E activity (20, 21). Much effort was devoted to unraveling the role of vitamin E during oxidative stress in animals. Similar to plants, it is assumed that the main vitamin E function in mammals includes the scavenging of reactive oxygen species. Vitamin E deficiency in rats leads to infertility and fetal death (22). However, similar to the plant system, no clear evidence was obtained for an *in vivo* role of tocopherol during oxidative stress.

High-throughput biochemical screening methods have successfully been used to isolate plant mutants affected in lipid biosynthesis (e.g., refs. 23 and 24). The availability of such mutants was the basis for the isolation of the corresponding genes by chromosome walking (e.g., refs. 25 and 26). To elucidate the function of tocopherol during the oxidative stress response pathway, we initiated a screening approach for *Arabidopsis* plants carrying mutations in tocopherol biosynthesis. Employing a biochemical, TLC-based screening procedure, we were able to isolate a mutant totally devoid of tocopherol.

Materials and Methods

Isolation and Molecular Characterization of a Tocopherol-Deficient Mutant. *Arabidopsis thaliana* (ecotype Col-2) M₂ plants derived from ethyl methanesulfonate mutagenesis were grown on soil under standard conditions (120 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 20°C, 60% air humidity, 16 h light/8 h dark). To induce the stress-related synthesis of lipids, leaves detached from individual plants were incubated overnight on a wet filter paper at 37°C. For screening, lipids were isolated from stressed leaves with 1 M KCl/0.2 M H₃PO₄ and diethylether, separated by TLC on silica plates with hexane/diethylether/acetic acid (90:10:1, vol/vol), and stained with iodine.

The *vte1* mutant was four times back-crossed to WT Col-2 to reduce the number of background mutations. Genomic DNA was isolated from F₂ plants derived from a cross to WT Landsberg *erecta* and used for PCR marker mapping (27, 28). The gene At4g32770 on chromosome 4 (BAC F4D11, GenBank accession no. AL022537) was amplified by PCR from *vte1* with the oligonucleotides PD202 (AAA GGA AGG ATT TGT TTT

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: DMGQ, 2,3-dimethyl-5-geranylgeranyl-1,4-hydroquinone; DMPQ, 2,3-dimethyl-5-phytyl-1,4-hydroquinone.

†To whom reprint requests should be addressed. E-mail: doermann@mpimp-golm.mpg.de.

GTT TGC GAC TG) and PD203 (GGA CCG AAC GAA CTA AAA TCA TAC ATA CA) and used for sequencing.

Northern Analysis, Heterologous Expression in *Escherichia coli*. For Northern analysis, total RNA was isolated from leaves of WT and *vte1* by using standard protocols (29, 30). The blot was hybridized to a ³²P-labeled At4g32770 probe obtained by PCR amplification from genomic *Arabidopsis* WT DNA with the primers PD202 and PD203.

The mature part of the protein without the apparent signal peptide (31) was amplified by PCR from first-strand cDNA, using the oligonucleotide primers PD224 (TAA TGG ATC CAC TCC GGA CTC CTC ACA GTG G) and PD225 (GCT CAA GCT TTT ACA GAC CCG GTG GCT TGA A). The PCR fragment was ligated into the *Bam*HI and *Hind*III sites of pQE31 and transferred into *E. coli* M15(pREP4) cells for protein expression (Qiagen, Hilden, Germany).

Enzyme Assay. Tocopherol cyclase activity was measured with 2,3-dimethyl-5-phytyl-1,4-hydroquinone (DMPQ) or 2,3-dimethyl-5-geranylgeranyl-1,4-hydroquinone (DMGQ) (32). The tocopherol cyclase reaction with these substrates resulted in the synthesis of γ -tocopherol and γ -tocotrienol, respectively (32). Reaction products were separated by HPLC (Waters 2690) on a RP30 column (Bischoff Chromatography, Leonberg, Germany; 230 \times 4.6 mm, 3.0 μ m) with isocratic elution in 100% methanol and detected by fluorescence (excitation at 295 nm; emission at 332 nm).

Tocopherol Analysis. For GC/MS analysis, *Arabidopsis* leaves were homogenized in liquid nitrogen and lipid extracted with 100 μ l of 1 M KCl/0.2 M H₃PO₄ and 400 μ l of diethylether. The organic phase was removed and the solvent evaporated in a nitrogen gas stream. After chemical modification with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide, the trimethylsilyl derivatives of lipids were analyzed by GC/MS as described (33). Tocopherol was quantified by fluorescence HPLC with standards (34).

Measurement of Chlorophyll and Chlorophyll Fluorescence. Chlorophyll was extracted from leaves with 80% (vol/vol) acetone and quantified by measuring the absorbances at 646 and 663 nm (35). *In vivo* chlorophyll fluorescence was determined with a pulse-amplitude modulation fluorimeter (PAM-2000, Heinz Walz, Effeltrich, Germany). The quantum yield was calculated from $(F_m' - F_t)/F_m'$, where F_t and F_m' are the fluorescence emissions of a light-adapted leaf under measuring light or after application of a saturating light pulse, respectively (36).

Results

Isolation of a Mutant Deficient in Tocopherol Synthesis. A genetic approach was chosen to further dissect the pathway of tocopherol synthesis. Different neutral lipids (free fatty acids, triacylglycerols, tocopherols) were found to accumulate during oxidative stress in leaves of higher plants (refs. 11–13; Fig. 1). The increase in tocopherol was particularly prominent, because its amount in leaves exposed to stress was up to 5 times higher as compared with control conditions. Based on these findings, a screening procedure for *Arabidopsis* mutants affected in tocopherol synthesis was developed. Detached leaves from single *Arabidopsis* plants were incubated overnight at 37°C resulting in a combination of different stress factors (i.e., heat, wounding). After screening 2,000 individual *Arabidopsis* M₂ mutant plants for changes in lipid composition by TLC, a tocopherol-deficient line was identified. This line was tentatively designated *vte1* (for vitamin E deficient).

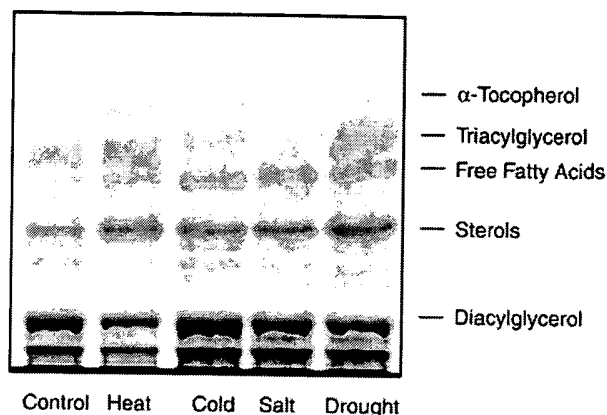


Fig. 1. Accumulation of neutral lipids during stress conditions in leaves. *Arabidopsis* WT plants were exposed to different stress conditions (heat, 3 days at 37°C; cold, 5 days at 4°C; drought, 3 days without watering; salt, watering with 0.5 M NaCl for 3 days). Lipids were extracted from leaves, separated by TLC, and stained with iodine.

The *vte1* Mutant Is Deficient in All Four Forms of Tocopherol and Contains Reduced Activity of Tocopherol Cyclase.

To unravel the biosynthetic block in the *vte1* mutant, lipids were isolated from WT and mutant leaves and analyzed by GC/MS after trimethylsilylation (Fig. 2A). In contrast to WT, which contained mostly α -tocopherol and small amounts of the other three forms of tocopherol (37), the *vte1* mutant was completely devoid of all four forms of tocopherol. However, in *vte1*, a compound with a molecular mass of 560.5 Da accumulated that was not detectable in WT (Fig. 2A). The mass spectrum of this compound is in agreement with the fragmentation pattern of the di(trimethylsilyl) derivative of DMPQ (Fig. 2B). This finding suggests that the *vte1* mutation affects the activity of DMPQ cyclase (tocopherol cyclase), because this enzyme catalyzes the conversion of 2-methyl-6-phytyl-1,4-hydroquinone and DMPQ to δ - and γ -tocopherol, respectively (Fig. 2C). Because these two forms of tocopherol are the precursors for β - and α -tocopherol, a reduction in tocopherol cyclase activity is expected to result in the loss of all four forms of tocopherol.

Enzyme activity for tocopherol cyclase was measured in leaf extracts from WT and *vte1* (Fig. 3A and B). Because enzyme activities in plant extracts using DMPQ or DMGQ were shown to be very similar (ref. 32 and data not shown), only one of these substrates, DMGQ, was used. Whereas in WT, a large amount of DMGQ was converted to γ -tocotrienol, the tocopherol cyclase activity was very low in *vte1* (407 ± 20 and 6.6 ± 0.7 ng of γ -tocotrienol per mg of protein per h for WT and *vte1*, respectively). The activity for 2-methyl-6-phytyl-1,4-hydroquinone methyltransferase was very similar in *vte1* (data not shown). Therefore, the *vte1* mutation specifically affects the activity of tocopherol cyclase and does not cause a general down-regulation of the enzymes of tocopherol biosynthesis.

Isolation of the *VTE1* Gene. F₂ plants derived from a cross of *vte1* with WT Landsberg *erecta* were used for mapping the mutation to the *Arabidopsis* genome. The *VTE1* locus was localized to the lower arm of chromosome 4, between the markers RSW1 and CAT2a (Fig. 4A). These two markers span a region of 10⁶ bp on the *Arabidopsis* genome. In this region, no genes encoding enzymes of tocopherol biosynthesis were found (37–41). Therefore, the *vte1* locus represents a novel gene involved in tocopherol biosynthesis. The gene At4g32770 on BAC F4D11 (GenBank accession no. AL022537) represented a candidate gene for *VTE1*, because it encodes a protein similar to the ORF slr1737

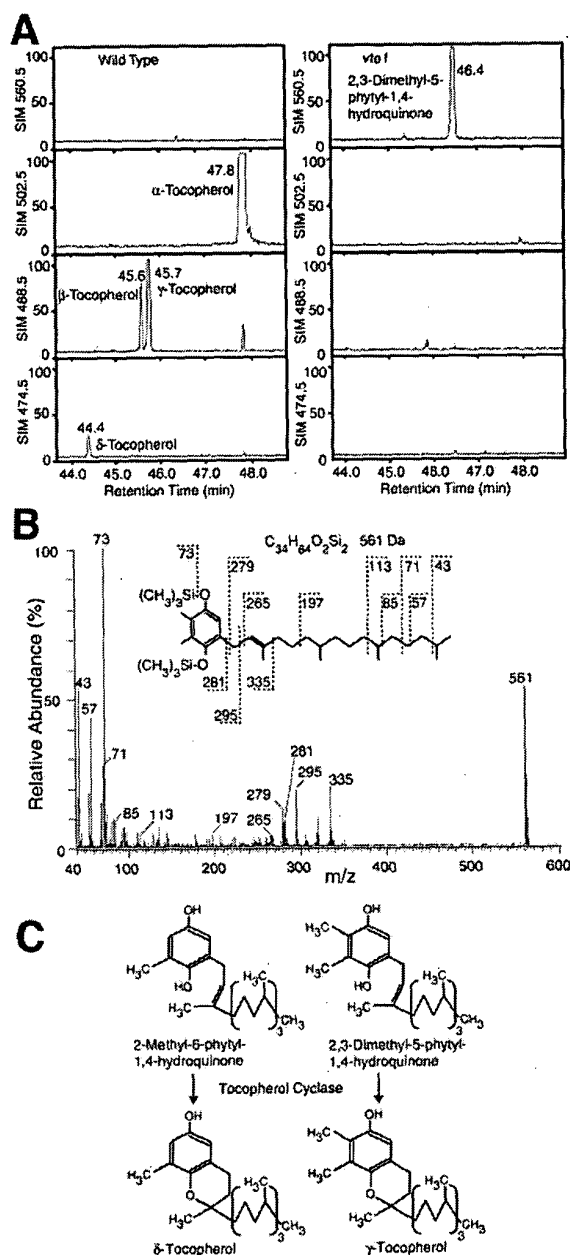


Fig. 2. All four forms of tocopherol are absent from *vte1* leaves. (A) GC/MS chromatograms of trimethylsilylated lipids from *Arabidopsis* WT and *vte1* leaves. For each sample (WT or *vte1* mutant), four single ion monitoring traces (SIM; m/z = 560.0, 502.5, 488.5, and 474.5) derived from one GC/MS chromatogram are presented. The signals are drawn to the same relative scale (100%). (B) The compound with the retention time t_r = 46.4 min accumulating in *vte1* leaves was identified as the di(trimethylsilyl) derivative of DMPQ based on its fragmentation pattern. (C) Conversion of 2-methyl-6-phytyl-1,4-hydroquinone or DMPQ by tocopherol cyclase yields δ - and γ -tocopherol, respectively.

of *Synechocystis* (52.2% and 41.1% identity on DNA and amino acid level, respectively). In *Synechocystis*, *slr1737* is localized in the same operon as *slr1736*, which encodes the homogentisate

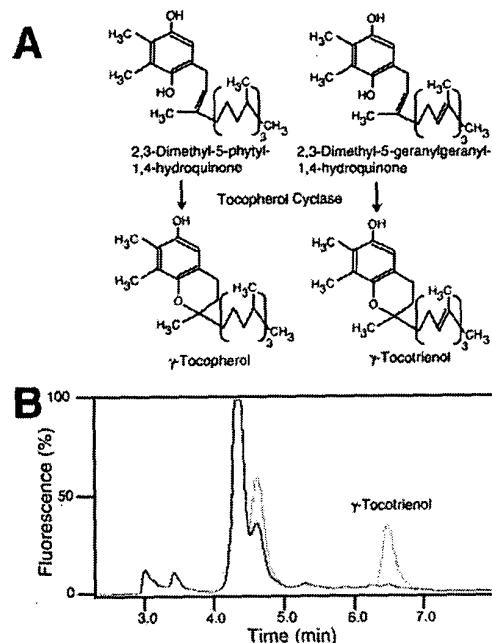


Fig. 3. The *vte1* mutant is deficient in tocopherol cyclase activity. (A) Tocopherol cyclase catalyzes the conversion of DMPQ and 2,3-dimethyl-5-geranylgeranyl-1,4-hydroquinone (DMGQ) to γ -tocopherol and γ -tocotrienol, respectively. (B) Tocopherol cyclase assays of WT and *vte1*. Protein extracts were incubated with DMGQ, and the reaction products separated by HPLC and detected by fluorescence emission. The retention time of γ -tocotrienol was confirmed with a standard. Dotted line, WT; solid line, *vte1*.

phytyltransferase, another enzyme of tocopherol synthesis (38, 39). Because in prokaryotes, genes encoding enzymes of the same biosynthetic pathway are oftentimes combined in operons, the ORF *slr1737* was a likely candidate for another gene of tocopherol synthesis. A similar genomics-based approach has already successfully been used to isolate the structural gene for γ -tocopherol methyltransferase (37). For this reason, the gene *At4g32770* from *Arabidopsis* was amplified by PCR from genomic DNA of *vte1* and completely sequenced. The sequence of the mutant gene showed only one mismatch as compared with WT at the splicing site of intron 3/exon 4. The last base at the 3' end of intron 3 of *At4g32770* was changed from G to A (Fig. 4B). By comparing 97 intron sequences from dicots, the G at this position was found to be 100% conserved (42). Therefore, it is very likely that in *vte1*, splicing of intron 3 is abolished, which results in the synthesis of a truncated, inactive protein.

To test whether expression of *At4g32770* was altered in *vte1*, Northern analysis was done with RNA isolated from leaves. A band with a size of about 1.6 kb hybridized to the *At4g32770* probe in WT, but in *vte1* no band was detectable at this position (Fig. 4C). Therefore, transcription of *At4g32770* or mRNA stability was strongly reduced in *vte1*.

VTE1 Encodes an Enzyme with Tocopherol Cyclase and Tocotrienol Cyclase Activity. To provide further evidence that the VTE1 protein of *Arabidopsis* codes for a functional enzyme with tocopherol cyclase activity, the corresponding cDNA was heterologously expressed in *E. coli* and used for enzyme assays. The protein contains an apparent N-terminal signal sequence for the chloroplast (ref. 31; see below). Therefore, only the mature part of the protein lacking the N-terminal 74 aa was used for *E. coli* expression. As depicted in Fig. 5, the VTE1 protein showed high

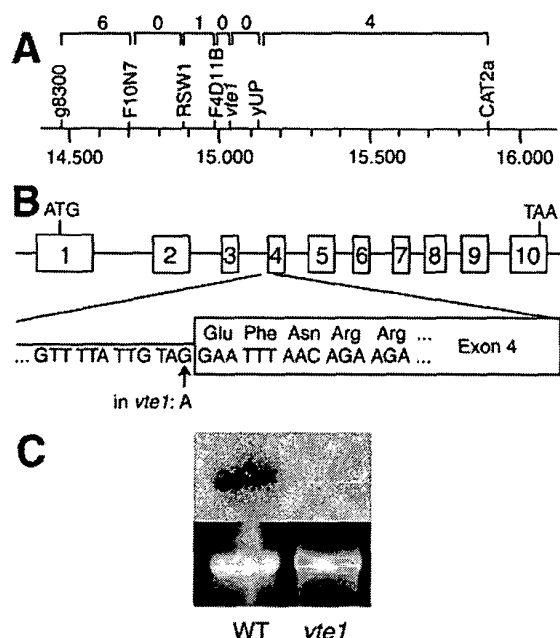


Fig. 4. Isolation of the *VTE1* gene. (A) Mapping of the *vte1* mutation to the lower arm of chromosome 4. The values on top represent the numbers of recombinations between two adjacent markers in a mapping population of 384 chromosomes. The numbers at the bottom indicate the position on the physical map of chromosome 4 in 10^6 bp according to the MIPS database (<http://mips.gsf.de/proj/thal/>). (B) Exon/intron structure of the gene At4g32770. In *vte1*, this gene carries a G to A point mutation at the 3' border of intron 3. (C) Northern analysis of total RNA from WT and *vte1*. (Upper) The hybridization signal with At4g32770. (Lower) The ethidium bromide-stained 25S rRNA bands.

tocopherol cyclase activity with the substrates DMPQ and DMGQ, resulting in the synthesis of γ -tocopherol and γ -tocotrienol, respectively. These findings provide clear evidence that

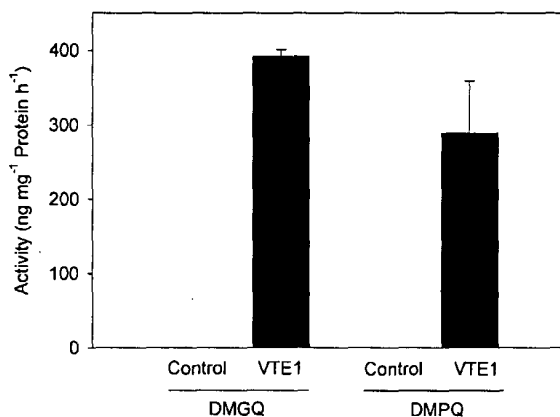


Fig. 5. Tocopherol cyclase activity of VTE1 after heterologous expression in *E. coli*. The mature part of the *VTE1* cDNA was expressed in *E. coli* and used for tocopherol cyclase activity assays with DMGQ or DMPQ. The products, γ -tocotrienol and γ -tocopherol, respectively, were quantified by fluorescence HPLC. The values represent the means of two independent measurements and standard errors. Control, *E. coli* transformed with empty vector (pQE31); VTE1, *E. coli* transformed with *VTE1* in pQE31. The tocopherol cyclase activity of control cells was below 1 ng per mg of protein per h for the two substrates.

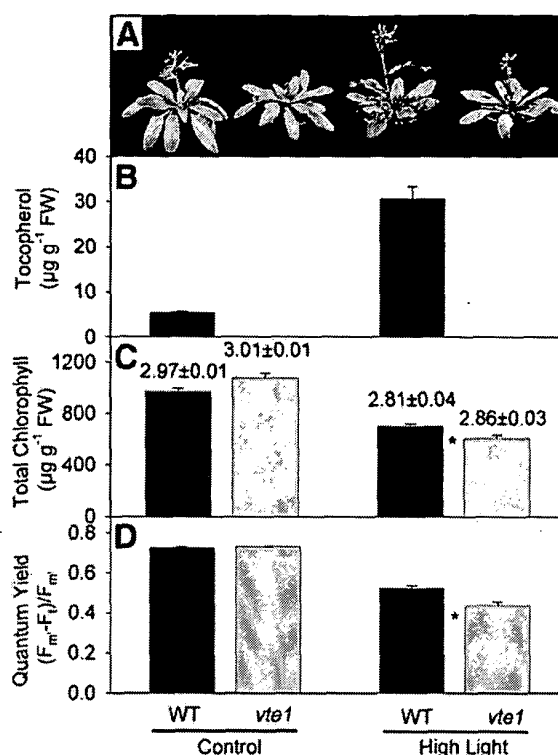


Fig. 6. Stress-induced changes in plant morphology, tocopherol, chlorophyll, and chlorophyll fluorescence. Thirty-five-day-old plants were transferred to high light ($850 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) for 5 days. (A) Visible phenotype of plants. (B) Total tocopherol content measured by fluorescence HPLC. (C) Total chlorophyll content. The numbers indicate the chlorophyll a to chlorophyll b ratio. (D) Photosynthetic quantum yield. Data represent the means \pm SE of three (B), 10 (C), or 30 (D) measurements each. Values for chlorophyll content and quantum yield that were different between WT and mutant as judged by Student's *t* test ($P < 0.02$) are indicated with an asterisk.

VTE1 encodes tocopherol cyclase and that this enzyme is involved in the synthesis of both tocopherols and tocotrienols.

The *vte1* Mutant Phenotype During Oxidative Stress. When raised under optimal growth conditions, the overall stature, chlorophyll content, and photosynthetic yield of WT and *vte1* plants were very similar (Fig. 6). The fresh weight of 50-day-old *vte1* plants was reduced by only 10–15%. The flowering time as determined from the number of rosette leaves at the time of the opening of the first flower (43) was similar in WT (16.9 ± 0.9 ; mean \pm SE, $n = 10$) and *vte1* (18.1 ± 1.1). Therefore, under optimal growth conditions, absence of all forms of tocopherol had no large impact on photosynthesis, growth, and development of the mutant plant. We considered the possibility that tocopherol deficiency might result in a decrease of photosynthetic efficiency only during oxidative stress. Plants were exposed to high-light conditions ($850 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and used for measurements of tocopherol, chlorophyll content, and chlorophyll fluorescence. Exposure to high light resulted in the occurrence of necrotic areas on the leaves of the *vte1* mutant and WT (Fig. 6A). In contrast to WT, the stress-induced accumulation of anthocyanin in the mutant was much less pronounced (Fig. 6A). Photo-oxidative stress resulted in an increase in α -tocopherol in WT, but no tocopherol was detectable in *vte1* under these conditions (Fig. 6B). Total chlorophyll was slightly reduced in *vte1* leaves during oxidative stress (Fig. 6C), but the chlorophyll a to

chlorophyll b ratio was not changed. The photosynthetic quantum yield determined by measurement of chlorophyll fluorescence was lower for WT and *vte1* under high-light stress as compared with control conditions, and it was even further decreased in *vte1* (Fig. 6D). Therefore, under conditions of photo-oxidative stress, chlorophyll content and photosynthesis in *vte1* mutant plants were more severely affected as compared with WT. The differences in chlorophyll content and quantum yield for *vte1* under high light were also observed with a set of independently raised plants exposed to $1,050 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and the difference in chlorophyll content was found to be more pronounced in old leaves as compared with young leaves (data not shown).

In addition to high light, *vte1* mutant plants were also analyzed during different stress conditions—e.g., paraquat treatment, high temperature (5 days at 37°C), and cold treatment (5 days at 4°C) (data not shown). Growth, chlorophyll content, and chlorophyll fluorescence of *vte1* plants were always very similar to that of WT.

Discussion

The *vte1* mutant of *Arabidopsis* represents the first higher plant mutant carrying a block that specifically affects tocopherol synthesis. The reduction in tocopherol cyclase activity accompanied with the accumulation of its substrate, DMPQ, and a concomitant decrease in γ -tocopherol strongly suggest that the *vte1* mutant carries a mutation in the structural gene encoding tocopherol cyclase.

Several lines of evidence indicate that the gene At4g32770 on chromosome 4 of *Arabidopsis* encodes the enzyme with tocopherol cyclase activity: (i) The *vte1* mutation was mapped to an interval of 10^6 bp on chromosome 4 encompassing the At4g32770 locus; (ii) the orthologous gene from *Synechocystis*, *slr1737*, is localized in the same operon as homogentisate phytyltransferase, another gene of tocopherol synthesis (38, 39); (iii) At4g32770 from *vte1* carries a splicing site mutation at a position absolutely conserved in dicots; (iv) At4g32770 mRNA abundance and tocopherol cyclase activity were strongly reduced in *vte1*; (v) heterologous expression of At4g32770 in *E. coli* resulted in the synthesis of a protein with high tocopherol cyclase activity. Because the *vte1* mutant was four times back-crossed to WT, it is extremely unlikely that this plant carries another mutation in a gene involved in tocopherol synthesis. The point mutation found at the highly conserved splicing site of At4g32770 strongly suggests that *vte1* represents a null allele. This was confirmed by the finding that the amounts of all forms of tocopherol were reduced below detection limit.

Growth, chlorophyll content, and photosynthetic quantum yield of *vte1* under optimal conditions were very similar to WT. Similarly, the homogentisate phytyltransferase mutant of *Synechocystis* deficient in all forms of tocopherol was not reduced in growth or chlorophyll content (38). However, during photo-oxidative stress, chlorophyll content and quantum yield were decreased in *vte1* as compared with WT. This result is in agreement with the presumed role of tocopherol in protecting

photosynthetic complexes in thylakoids from oxidative stress. However, the stress-induced changes observed for *vte1* were rather small. Therefore, other redox systems (e.g., ascorbate, glutathione, carotenoids) might be involved in scavenging reactive oxygen species under high-light conditions.

The reduction in geranylgeranyl reductase activity by antisense expression in tobacco plants also resulted in a partial reduction of tocopherol synthesis (44, 45). Similar to the *Arabidopsis vte1* mutant, only under conditions of oxidative stress, photosynthesis in the transgenic tobacco lines was affected. However, in contrast to *vte1*, which is exclusively affected in tocopherol synthesis, chlorophyll biosynthesis in the geranylgeranyl reductase antisense plants was also reduced. Therefore, it was not clear whether the phenotypical changes observed for the tobacco antisense lines were derived from deficiency in tocopherol or chlorophyll. Similarly to *vte1*, an *Arabidopsis* mutant deficient in ascorbate synthesis (*soz1/vtc1*) showed an increased sensitivity to environmental stress (46). The two mutants, *vte1* and *vtc1*, open the way to study the *in vivo* role of both vitamin E and vitamin C during oxidative stress in higher plants.

Interestingly, *VTE1* from *Arabidopsis* shows high sequence similarity (62% identity on amino acid level) to the maize *SXD1* gene (31, 47). The findings that only one *VTE1*-like locus is found in the *Arabidopsis* genome and that in maize all *SXD1*-like expressed sequence tags present in the public database have a sequence identical to *SXD1* suggest that *VTE1* and *SXD1* represent (orthologous) single copy genes, both encoding enzymes with the same function—i.e., tocopherol cyclase activity. The *sucrose export defective mutant1* of maize (*sxd1*) shows alterations in the ultrastructure of plasmodesmata, which was implied to be the cause for the reduced photoassimilate export from mutant leaves. The *VTE1/SXD1* cDNA was predicted to encode a hydrophilic protein of 488 aa (54.7 kDa) with an N-terminal plastid signal sequence (31). BLAST searches in GenBank revealed the presence of expressed sequence tags in many different plant species, but no similarities to animal or yeast sequences. The fact that the *VTE1/SXD1* protein was imported into pea chloroplasts (47) is in agreement with the presumed localization of tocopherol cyclase to the inner chloroplast envelope (19). During import experiments, the pea protein was processed indicating the presence of a cleavable N-terminal signal peptide. It was proposed that *VTE1/SXD1* represents a protein factor involved in chloroplast-to-nucleus signaling of the metabolic status of chloroplasts (31). The finding that *VTE1/SXD1* encodes an enzyme of tocopherol synthesis raises the question of the exact nature of the factor(s) responsible for the morphological and metabolic changes observed for the maize *sxd1* mutant. Therefore, comparative experiments with the two mutants, *sxd1* from maize and *vte1* from *Arabidopsis*, are required to clarify the role of lipid antioxidants during oxidative stress, as well as for primary reactions of photosynthesis and photoassimilate transport.

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